IDENTIFICATION OF
2α-HYDROXY-4-PREGNENE-3,20-DIONE IN HUMAN
PREGNANCY URINE

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Summary—2α-Hydroxyprogesterone (2α-hydroxy-4-pregnene-3,20-dione) was identified in
human late pregnancy urine by liquid-gel chromatography, GLC and GC–MS. In addition,
the following 2-hydroxylated C_{21} steroids were found and identified as 2ξ-hydroxy-5ξ-preg-
nane-3,20-dione, 2ξ,20ξ-dihydroxy-4-pregnen-3-one, 2α,3α-dihydroxy-5α- (and 5β)-pregnan-
20-one, two isomers of pregnane-2,3,20-triol and 2ξ,3ξ,16ξ-trihydroxy-5ξ-pregnan-20-one.

INTRODUCTION

During human gestation the known principal
pathway for the elimination of steroids is
urinary secretion. Much research has been done
over several years to isolate and identify the
major urinary progesterone metabolites. These
compounds, however, account for only 10–30%
of all progesterone metabolites [1]; therefore
several quantitatively minor steroids still remain
to be identified. This paper reports the identifi-
cation of 2α-hydroxyprogesterone and of other
2-hydroxylated C_{21} steroids found in the urine of
a woman during the third trimester of gestation.

EXPERIMENTAL

Subject

24-h urine samples (1680 ml) were collected
from a normal pregnant woman in week 39 of
gestation. The urine was stored at −20°C until
analyzed. This patient presented a normal preg-
nancy and delivered a mature, male live-born
baby.

Chemicals

All solvents (analytical grade) and anhydrous
sodium sulfate were purchased from Merck
(Darmstadt, Fed. Rep. Germany). *N-O*-
bis-(trimethylsilyl)-trifluoroacetamide (BSTFA)
was obtained from the Pierce Chemical Co.
(Rockford, IL, U.S.A.). Methoxylamine hydro-

chloride (Eastman Organic Chemicals,
Rochester, NY, U.S.A.) was used in a pyridine
solution (8 g/l). *Helix pomatia* digestive juice
was purchased from l'Industrie Biologique
Française (Gennevilliers, France). Sephadex
LH-20 was obtained from Pharmacia Fine
Chemicals (Uppsala, Sweden). The solvent mix-
ture used for Sephadex LH-20 chromatography
was chloroform–heptane–methanol (50:50:10,
v/v) [2].

Steroids

Except for 5α-cholestane obtained from
Makor Chemicals Ltd (Jerusalem, Israël), all
other reference steroids were purchased from
Steraloids Inc. (Wilton, NH, U.S.A.).

Liquid-gel chromatography

Glass columns, 30 × 0.4 cm (i.d.), with a
100 ml solvent reservoir and Teflon stopcock
were used. Sephadex LH-20 (1 g) was suspended
in 10 ml of chloroform–heptane–methanol. The
mixture was stirred for 15 min, added to the
column and left to stand overnight [2].

Procedure for the analysis of steroids in urine

70 ml of urine were used; briefly, the pH was
adjusted to 5.20 with acetic acid; sodium acetate
buffer (3 ml, pH 5.20, 0.1 M), *Helix pomatia*
digestive juice (1000 IU of β-glucuronidase per
ml of urine) were added and the sample
incubated at 37°C for 48 h. A further 1000 IU
of β-glucuronidase was added after the first
24 h. Following hydrolysis, the liberated

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steroids were extracted twice with ethyl acetate (1:1, v/v). The combined ethyl acetate phases were washed with sodium bicarbonate (1 M) and distilled water until neutral, dried by filtration on anhydrous sodium sulfate, and evaporated to dryness. The residue was dissolved by ultra-sonication in 0.2 ml of chloroform–heptane–methanol and the solution transferred to the Sephadex LH-20 column. The steroids were eluted with 60 ml of chloroform–heptane–methanol. Fractions of 0.5 ml were collected. Each fraction was evaporated to dryness under N_2. The residue was dissolved in methanol and divided in two parts. The first in order to obtain the steroid trimethylsilyl (TMS) ethers and the second to obtain the steroid O-methyloxime (MO) and O-methyloxime-trimethylsilyl (MO-TMS) ethers. The TMS ethers were acquired by dissolution of residue in 0.15 ml of pyridine–BSTFA (1:3, v/v) and left to stand overnight at 60°C. The MO derivatives were acquired using 0.2 ml of methoxylamine hydrochloride solution in pyridine. After 12 h at 60°C the solution was evaporated to dryness under N_2 and the MO-TMS ethers were obtained as above. The TMS ethers and MO-TMS ether derivatives were analyzed by GLC and GC–MS.

GLC and GC–MS

A Packard 427 gas chromatograph equipped with a SE-30 capillary column (25 m × 0.32 mm i.d.) and a flame ionization detector was employed. The carrier gas was helium. The temperatures of the injection port and detector were 270 and 280°C, respectively. The tempera-

ture was programmed from 180 to 280°C at the rate of 2°C/min. In addition each sample was analyzed under isothermal conditions (220°C).

GC–MS analysis was carried out using a Nermag R1010C instrument coupled with a Delsi gas chromatograph Model DI 700 which was equipped with a SE-30 capillary column (25 m × 0.32 mm i.d.). The carrier gas was helium. The ionizing energy was 70 eV. Temperatures were: ion source 180°C; injector 280°C; and interface to the mass spectrometer 270°C. The column was programmed from 200 to 290°C at a rate of 5°C/min. The mass spectra were collected and processed with a Digital PDP 11-23 data system.

Identification of steroid

A steroid was considered as identified when the methylene unit values (MU) on SE-30 and the mass spectrum were identical to those of the corresponding reference compound. Under isothermal conditions the retention times (t_R) of steroids are expressed relative to that of 5α-cholestane.

RESULTS AND DISCUSSION

GLC and GC–MS analysis of 2α-hydroxyprogesterone

The methylene unit values of 2α-hydroxyprogesterone TMS ether and MO-TMS ether were 28.98 and 29.21, respectively. The relative retention times were 1.43 for TMS ether and 1.54 for the MO-TMS derivative. The mass spectrum of 2α-hydroxyprogesterone TMS ether (Fig. 1)
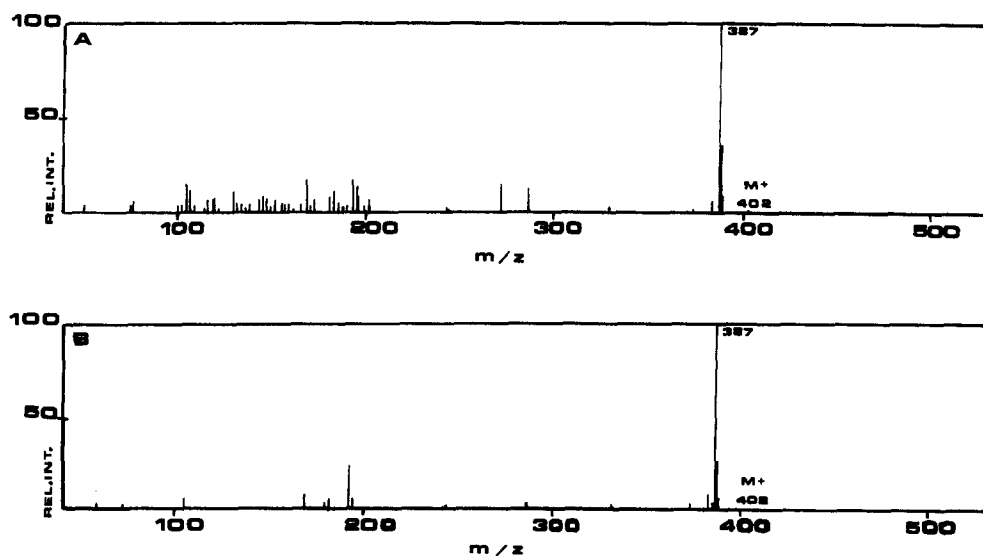

Fig. 1. Mass spectrum of reference 2α-hydroxyprogesterone TMS ether (A) and of the TMS ether of compound 6 (B).

gave a molecular ion (M) at m/z 402 and a base peak at m/z 387 (M-15). The mass spectrum of 2α-hydroxyprogesterone MO-TMS ether (Fig. 2) gave a molecular ion (M) at m/z 460, a base peak at m/z 429 (M-31) and prominent peaks at m/z 100, 339 [M-(31 + 90)], 355 [M-(15 + 90)], 358, 370 (M-90), 402 and 445 (M-15). The principal characteristics of these mass spectra have been discussed by Gustafsson and Lisboa [3].

GLC and GC–MS analysis of urinary steroids

Figure 3 shows the GC analysis of the steroid TMS ethers eluted in Sephadex LH-20 fraction 5. In this fraction seven distinct steroids were identified by GLC and GC–MS. They are 3α-hydroxy-5α-androstan-17-one(compound 1), 3α-hydroxy-5β-androstan-17-one (compound 2), 3α-hydroxy-5β,17α-pregnan-20-one (compound 3), 3α-hydroxy-5α-pregnan-20-one (compound 4), 3α-hydroxy-5β-pregnan-20-one (compound 5) and 5-cholesten-3β-ol (compound 7). The mass spectra of the TMS ether (MU:28.97; t_R:1.44) and MO-TMS (MU:29.22; t_R:1.54) derivatives of compound 6 are shown in Figs 1 and 2, respectively. They were identical to the TMS ether and MO-TMS derivatives of the reference compound. According to GC–MS data compound 6 was identified as 2α-hydroxyprogesterone. The TMS ether mass spectrum of another steroid (compound 8) showed a fragmentation pattern very similar to that of 2α-hydroxyprogesterone, the only difference being that the molecular ion (M) at m/z 404 and the base peak at m/z 389 (M-15) were

2 mass units higher than the former mass spectrum. Therefore this compound, revealed only by GC–MS, was tentatively identified as 2ξ-hydroxy-5ξ-pregnane-3,20-dione.

During this study, we also found six other 2-hydroxylated C_{21} steroids (compounds 9 to 14).

Compound 9. The mass spectrum of the MO-TMS ether derivative of this compound is similar to that of 2α,20α-dihydroxy-4-pregnen-3-one [4]. It gave a molecular ion (M) at m/z 505, a base peak at m/z 117 and prominent peaks at m/z 490 (M-15) and 474 (M-31). On the basis of these data, compound 9 was tentatively identified as 2ξ,20ξ-dihydroxy-4-pregnen-3-one. It would seem that the mass spectrum of the TMS ether of the latter compound was that found to have prominent peaks at m/z 117 and m/z 461 (base peak). Molecular ion was recorded at m/z 476.

Compounds 10 and 11. The TMS ethers mass spectra of compounds 10 and 11 are quite identical and similar to those previously published for 2α,3α-dihydroxy-5α-pregnan-20-one [5]. The major fragments were found at m/z 478 (M), 463 (M-15), 388 (M-90), 299 [M-(90 + 89)], 281, 147 (base peak), 143, 142 and 129. The peaks at m/z 129, 142 and 143 indicate the presence of trimethylsilyloxy groups on C-2 and C-3 [6]. The origin of the fragment at m/z 147, particularly abundant in these mass spectra, has been discussed by several authors [7, 8]. The MO-TMS ether derivatives of compounds 10 and 11 yielded mass spectra characterized by a molecular ion (M) at m/z 507 and a base peak at m/z 100. The fragments at
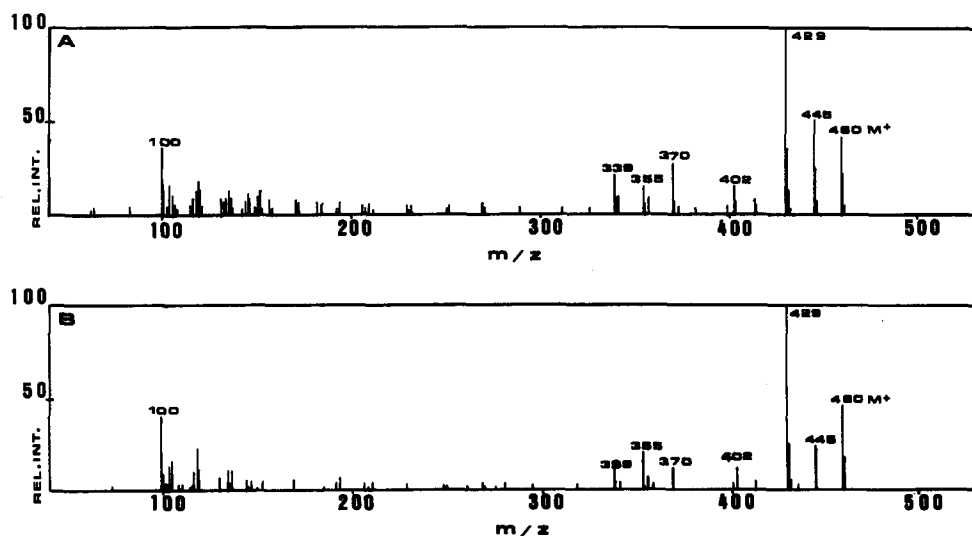

Fig. 2. Mass spectrum of reference 2α-hydroxyprogesterone MO-TMS derivative (A) and of the MO-MS derivative of compound 6 (B).
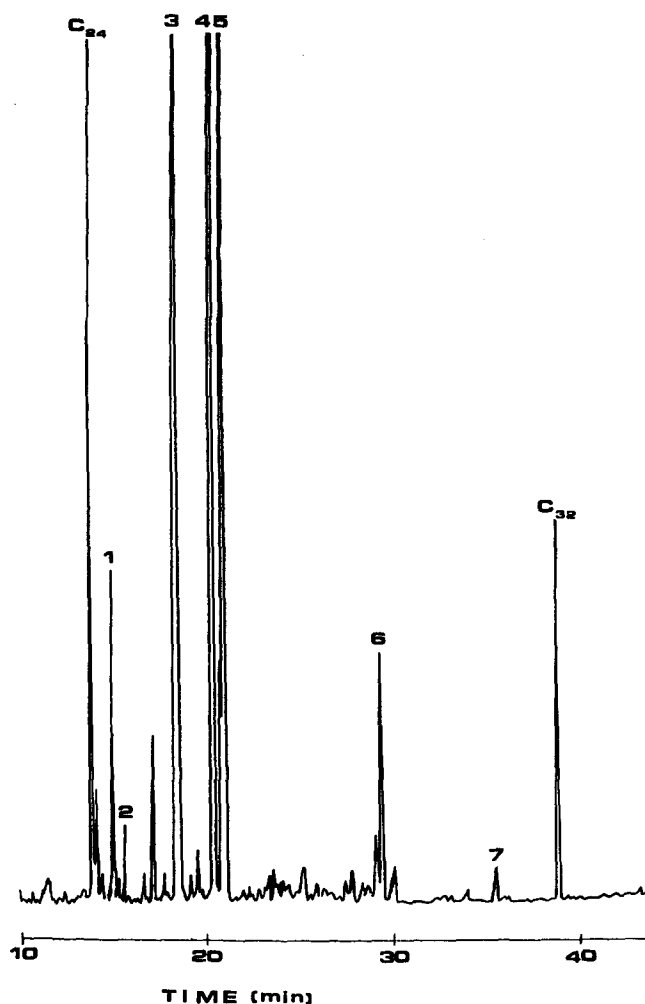
Fig. 3. Gas chromatographic analysis (SE-30 column) of silyl ethers of steroids isolated in the Sephadex LH-20 fraction 5 of urine from a normal pregnant woman. The numbered compounds were identified as the TMS ethers of 3α-hydroxy-5α-androstan-17-one (compound 1), 3α-hydroxy-5β-androstan-17-one (compound 2), 3α-hydroxy-5β,17α-pregnan-20-one (compound 3), 3α-hydroxy-5α-pregnan-20-one (compound 4), 3α-hydroxy-5β-pregnan-20-one (compound 5), 2α-hydroxy-progesterone (compound 6) and 5-cholesten-3β-ol (compound 7).

m/z 492 (M-15), 476 (M-31), 402 [M-(15 + 90)], 386 [M-(31 + 90)] and 296 [M-(31 + 2 × 90)] and those at 318, 328 and 331, were also found in these mass spectra [4]. Moreover the fragments at m/z 129, 142, 143 and 147 previously mentioned, were also observed.

The comparison of the GLC data of the TMS ether of compound 10 (Table 1) with those published [9, 10] shows this compound to be 2α,3α-dihydroxy-5α-pregnan-20-one. The GLC behavior of compound 11 on the one hand and its biological behavior on the other hand, allow us to conclude that this steroid is 2α,3α-dihydroxy-5β-pregnan-20-one.

Compounds 12 and 13. The mass spectra of the silyl ethers of compounds 12 and 13 gave a molecular ion (M) at m/z 552, a base peak at

m/z 117 and peaks at m/z 129, 142, 143 [6], 147, 462 (M-90), 537 (M-15). The peak at m/z 117 is typical of 20-trimethylsilyloxy-21-deoxy-steroids [11]. Therefore, compounds 12 and 13 were tentatively identified as 5-pregnane-2,3,20-triol isomers.

Compound 14. The TMS ether of compound 14 gave a molecular ion at m/z 566, indicating a pregnanetriolone structure. The peaks at m/z 96, 109, 157, 159, 172 and 186 are specific for a 16-trimethylsilyloxy-20-one configuration [12]. Peaks at m/z 129, 142, 143 were also found. Several prominent peaks were seen at m/z 551 (M-15), 476 (M-90), 461 [M-(90 + 15)], 386 [M-(2 × 90)], 371 [M-(15 + 2 × 90)], 297 [M-2 × 90 + 89)] and 147. Thus, compound 14 was identified as 2ξ,3ξ,16ξ-trihydroxy-5ξ-

Table 1. Gas chromatographic characteristics of MO-TMS and TMS ethers of steroids identified in Sephadex LH-20 fractions

| No.	Steroids	Elution volume (ml)	MU		t_R	
			TMS	MO-TMS	TMS	MO-TMS
1	3α-hydroxy-5α-androstan-17-one	2.0–2.5	24.35	25.12	0.37	0.45
2	3α-hydroxy-5β-androstan-17-one	2.0–2.5	24.55	25.32	0.39	0.48
3	3α-hydroxy-5β,17α-pregnan-20-one	2.0–2.5	25.45	25.22	0.51	0.47
4	3α-hydroxy-5α-pregnan-20-one	2.0–2.5	26.09	26.99	0.62	0.81
5	3α-hydroxy-5β-pregnan-20-one	2.0–2.5	26.25	27.14	0.65	0.84
6	2α-hydroxy-4-pregnene-3,20-dione	2.0–2.5	28.97	29.22	1.44	1.54
7	5-cholesten-3β-ol	1.5–2.5	30.94	—	2.52	—
8	2ξ-hydroxy-5ξ-pregnane-3,20-dione	2.5	—	—	—	—
9	2ξ,20ξ-dihydroxy-4-pregnen-3-one	4.0	—	—	—	—
10	2α,3α-dihydroxy-5α-pregnan-20-one	3.5–4.0	28.04	28.90–29.32[a]	1.08	1.40–1.58[a]
11	2α,3α-dihydroxy--5β--pregnan-20-one	4.5	28.10	28.65	1.11	1.33
12	5ξ-pregnane-2ξ,3ξ,20ξ-triol	5.5–7.0	29.40	—	1.61	—
13	5ξ-pregnane-2ξ,3ξ,20ξ-triol	7.5–8.0	29.31	—	1.57	—
14	2ξ,3ξ,16ξ-trihydroxy-5ξ-pregnan-20-one	6.5–9.0	29.71	30.41	1.78	2.21

[a]*Syn-* and *anti*-isomers.
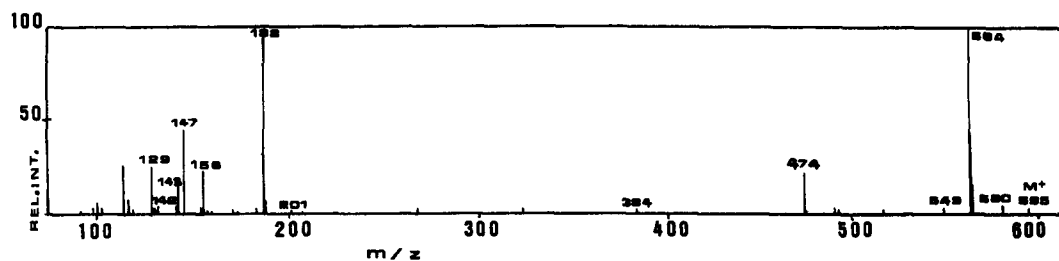
Fig. 4. Mass spectrum of the MO-TMS derivative of the compound 14 tentatively identified as 2ξ,3ξ,16ξ-trihydroxy-5ξ-pregnan-20-one. This steroid presents a molecular ion at m/z 595 and the base peak at m/z 564 (M-31). The proposed sequence of 2,3,16-trihydroxypregnan-20-one structure is suggested by the fragments at m/z 129, 142, 143, 147, 156, 188 and 201. The other fragments are observed at m/z 580 (M-15), 549 [M-(31 + 15)], 474 [M-(31 + 90)], 384 [M-(31 + 2 × 90)]

pregnan-20-one. The MO-TMS mass spectrum of this compound is shown in Fig. 4 [13].

Obviously, the accurate configuration of the last seven steroids could not be determined because the appropriate reference compounds were not available.

In this paper, the identification of 2α-hydroxyprogesterone in the urine of a woman 39 weeks pregnant is described. In this case about 100 μg/day of this compound was excreted. This steroid has previously been characterized by TLC and GC–MS after the incubation of progesterone with liver microsome preparations from adult male rats [3]. Except for the 2ξ,20ξ-dihydroxy-4-pregnen-3-one, the other 2-hydroxylated C₂₁ steroids mentioned in this work have previously been described in the faeces of a germfree rat after i.p. administration of [4-¹⁴C]pregnenolone [9], in isolated rat liver perfused with progesterone [14], and in the urine of pregnant women [15, 16]. The 2α-hydroxylation of pregnenolone [17], pregnanolone sulfate [5] and aldosterone [18] by rat liver microsomes has been demonstrated. Similarly, after incubation of cortisol with guinea pig liver microsomes the 2α-hydroxycortisol is formed [19].

The site and biological importance of 2α-hydroxylation of progesterone during human pregnancy have not yet been established. However, it appears from experimental data reviewed by Shackleton [20], Pasqualini and Kincl [21] that the human placenta lacks 2-hydroxylase activity and that this enzymatic activity is present in the fetal compartment [22]. Although the role of maternal liver cannot be excluded, we think that the fetal liver will be the principal site of 2α-hydroxylation of progesterone.

REFERENCES

1. Baillie T. A., Curstedt T., Sjövall K. and Sjövall J.: Production rates and metabolism of sulfates of 3β-hydroxy-5α-pregnane derivatives in pregnant women. *J. Steroid Biochem.* **13** (1980) 1473–1486.
2. Archambault A., Bègue R.-J., Faure Z. and Gandin B.: Chromatography of C₁₈, C₁₉ and C₂₁ steroids on Sephadex LH-20. *J. Chromat.* **284** (1984) 261–268.
3. Gustafsson J.-Å. and Lisboa B. P.: Studies on the metabolism of C₂₁ steroids in rat liver. Hydroxylation of

progesterone in rat liver microsomes. *Eur. J. Biochem.* **15** (1970) 525–530.

4. Ramirez L. C., Bournot P. and Maume B. F.: Bioconversion of 2α-hydroxyprogesterone to 2-hydroxylated corticosteroids by newborn rat adrenal cells in primary culture. *Biochim. Biophys. Acta* **847** (1985) 235–246.

5. Baillie T. A., Eriksson H., Herz J. E. and Sjövall J.: Specific deuterium labelling and computerized gas chromatography-mass spectrometry in studies on the metabolism *in vivo* of a steroid sulphate in the rat. *Eur. J. Biochem.* **55** (1975) 157–165.

6. Gustafsson J.-Å., Lisboa B. P. and Sjövall J.: Studies on the metabolism of C$_{19}$ steroids in rat liver. II. Biosynthesis of hydroxylated derivatives of 17β-hydroxy-5α-androstan-3-one in rat liver microsomes. *Eur. J. Biochem.* **5** (1968) 437–443.

7. Sloan S., Harvey D. J. and Vouros P.: Interaction and rearrangement of trimethylsilyloxy functional groups. The structural significance of the m/e 147 ion in the mass spectra of trimethylsilyl steroidal ethers. *Org. Mass Spectrom.* **5** (1971) 789–799.

8. Brooks C. J. W., Harvey D. J., Middleditch B. S. and Vouros P.: Mass spectra of trimethylsilyl ethers of some Δ^5-3β-hydroxy C$_{19}$ steroid. *Org. Mass Spectrom.* **7** (1973) 925–948.

9. Eriksson H. and Gustafsson J.-Å.: Steroids in germfree and conventional rats. Steroids in the mono- and disulphate fractions of faeces from female rats. *Eur. J. Biochem.* **16** (1970) 252–260.

10. Eriksson C. G. and Eneroth P.: Studies on rat liver microsomal steroid metabolism using ^{18}O-labelled testosterone and progesterone. *J. Steroid Biochem.* **28** (1987) 549–557.

11. Sjövall J. and Vihko R.: Identification of 3β,17β-dihydroxy-androst-5-ene, 3β,20α-dihydroxy-pregn-5-ene and epiandrosterone in human peripheral blood. *Steroids* **7** (1966) 447–458.

12. Gustafsson B. E., Gustafsson J.-Å. and Sjövall J.: Steroids in germfree and conventional rats. 2. Identification of 3α,16α-dihydroxy-5α-pregnan-20-one and related compounds in faeces from germfree rats. *Eur. J. Biochem.* **4** (1968) 568–573.

13. Desgres J.: La 6α-hydroxylation, marqueur du métabolisme foetal et néonatal de la progestérone dans le foie in vivo et dans les cultures de cellules épithéliales hépatiques. Doctorat es Sciences d'Etat, Faculté des Sciences, Université de Dijon, (1981) p. 91.

14. Eriksson H., Gustafsson J.-Å. and Pousette Å.: Metabolism of androstenedione and progesterone in the isolated perfused rat liver. *Eur. J. Biochem.* **27** (1972) 327–334.

15. Bègue R.-J., Desgres J., Gustafsson J. Å. and Padieu P.: Analyse modulaire des stéroïdes urinaires pendant la gestation humaine. *J. Steroid Biochem.* **7** (1976) 211–221.

16. Bègue R.-J., Morinière M. and Padieu P.: Urinary excretion of 5β-pregnane-3α,6α,20α-triol in human gestation. *J. Steroid Biochem.* **9** (1978) 779–784.

17. Danielsson H. and Johansson G.: 2-Hydroxylation of pregnenolone by rat liver microsomes. *FEBS Lett.* **25** (1972) 329–333.

18. Latif S. A., Morris D. J., Wei L., Kirk D. N., Burke P. J., Toms H. C. and Shackleton C. H. L.: 18-Substituted steroids-part 17. 2α-hydroxylated liver metabolites of aldosterone identified by high-field [^{1}H]NMR spectroscopy. *J. Steroid Biochem.* **33** (1989) 1119–1125.

19. Burstein S.: Determination of initial rates of cortisol 2α- and 6β-hydroxylation by hepatic microsomal preparation in guinea pig. Effect of phenobarbital in two genetic types. *Endocrinology* **82** (1968) 547–554.

20. Shackleton C. H. L.: Steroid synthesis and catabolism in the fetus and neonate. In *Biochemistry of Steroid Hormones* (Edited by H. L. J. Makin). Blackwell, Oxford (1984) pp. 441–477.

21. Pasqualini J. R. and Kincl F.: *Hormones and the Fetus*. Pergamon Press, Oxford, Vol. I (1985) p. 146.

22. Lisboa B. P.: Metabolism of neutral steroids in the human foetus investigated by *in vitro* studies. In *Hormonal Steroids* (Edited by V. H. T. James and L. Martini). Excerta Medica, Amsterdam (1970) pp. 511–521.